Virology 405 (2010) 149–156

Contents lists available at ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

# Domain disruptions of individual 3B proteins of foot-and-mouth disease virus do not alter growth in cell culture or virulence in cattle

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ARTICLE INFO

*Article history:*
Received 15 January 2010
Returned to author for revision
23 February 2010
Accepted 27 May 2010
Available online 30 June 2010

*Keywords:*
Foot-and-mouth disease
Protein 3B
Mutagenesis
Cattle

ABSTRACT

Picornavirus RNA replication is initiated by a small viral protein primer, 3B (also known as VPg), that is covalently linked to the 5' terminus of the viral genome. In contrast to other picornaviruses that encode a single copy of 3B, foot-and-mouth disease virus (FMDV) encodes three copies of 3B. Viruses containing disrupted native sequence or deletion of one of their three 3B proteins were derived from a FMDV A24 Cruzeiro full-length cDNA infectious clone. Mutant viruses had growth characteristics similar to the parental virus in cells. RNA synthesis and protein cleavage processes were not significantly affected in these mutant viruses. Cattle infected by aerosol exposure with mutant viruses developed clinical disease similar to that caused by the parental A24 Cruzeiro. Therefore, severe domain disruption or deletion of individual 3B proteins in FMDV do not affect the virus' ability to replicate *in vitro* and cause clinical disease in cattle.

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## Introduction

Foot-and-mouth disease (FMD) is a highly contagious viral disease of cattle, pigs, sheep, goats, and wild cloven-hoofed animals. The disease is characterized by fever and vesicular lesions of the epithelium of the mouth, tongue, feet, and teats (Alexandersen et al., 2003). The causal agent, FMD virus (FMDV), is a positive-stranded RNA virus that is the type species of the Aphthovirus genus of the *Picornaviridae* family.

The 8-kb FMDV genome is involved in translation and in replication. During replication, the genome is expressed as a single open reading frame (ORF) that is processed into mature polypeptide products. Translation of the ORF begins with a proteinase (Lpro), which is followed by the structural proteins of the P1 region (1A, 1B, 1C, and 1D) and the remaining nonstructural proteins of the P2 (2A, 2B and 2C) and P3 regions (3A, 3B, 3Cpro, and 3Dpol) (Grubman and Baxt, 2004). The 3C protease is responsible for most of the cleavages during the FMDV polyprotein processing (Vakharia et al., 1987), and 3Dpol is the viral RNA-dependent RNA polymerase (Grubman and Baxt, 2004). Protein 3B is covalently bound to the 5′ end of the genome and antigenome, and functions in priming picornavirus RNA synthesis (Wimmer, 1982). In addition to the four terminal P3 cleavage products (3A, 3B, 3C, and 3D proteins) and the uncleaved P3 polyprotein, several "intermediates" are observed in infected cells (3AB, 3CD, and 3BCD proteins) (Oh et al., 2009). As reviewed by Oh et al. (2009), it has been shown for poliovirus that these intermediate cleavage products of the P3 region can have different functions from their terminal cleavage products (3A, 3B, 3C and 3D).

In FMDV, 3B is present in three similar but nonidentical copies ($3B_1$, $3B_2$ and $3B_3$) (Forss and Schaller, 1982) that are 23 or 24 amino acids in length (King et al., 1980). Although not all three copies of FMDV 3B are needed to maintain infectivity (Falk et al., 1992; Pacheco et al., 2003), there are no reports of naturally occurring FMDV strains with fewer than three copies of 3B, suggesting that there is a strong selective pressure toward maintaining this redundancy (Carrillo et al., 2007; MacKenzie et al., 1975). This is an unusual finding, since FMDV is known to readily undergo homologous recombination to remove redundant genetic material (King et al., 1982). However, it has been reported that deletion of the $3B_3$ coding sequence within the context of the full-length infectious cDNA resulted in the production of a noninfectious RNA transcript (Falk et al., 1992). Additionally, $3B_3$ seems to be the most efficient substrate for the FDMV 3Dpol activity when each of the FMDV 3Bs is uridylylated *in vitro* (Nayak et al., 2005). It has been described that the loss of $3B_3$ sequence may also have a deleterious effect on FMDV RNA replication (Nayak et al., 2005). Laboratory-generated viruses lacking the first two 3B peptides were impaired in their ability to replicate in porcine cells in culture and caused attenuated disease in pigs showing that the presence of three 3Bs appear to control the virus' pathogenic potential and host range (Pacheco et al., 2003).

In this work we analyzed the *in vitro* and *in vivo* characteristics and pathogenic potential of viruses derived from a FMDV A24 Cruzeiro full-length cDNA infectious clone (A24-WT) containing modified forms or deletion of individual 3B peptides. Three viruses were created carrying an in-frame 57-nucleotide (19 amino acid) insertion randomly located into

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0042-6822/$ – see front matter. Published by Elsevier Inc.
doi:10.1016/j.virol.2010.05.036

one of the three different 3Bs resulting in severe disruption of the native amino acid sequence of the target proteins. Two additional viruses were also obtained which eliminated not only the 57 nucleotide transposon insertion after transfection but also had a partial deletion of the 3B region (3′ end of $3B_1$ and 5′ end of $3B_2$, in both cases). Interestingly, our results demonstrated that all 5 mutant viruses grew similarly, showed similar RNA replication kinetics and had similar plaque size in cell culture to those of the parental virus. Additionally, cattle infected by aerosol exposure with each of the five viruses developed infection and clinical disease similar to that caused by the parental FMDV A24-WT. Therefore, we provide evidence that the introduction of significant individual domain alterations on each of the 3B proteins do not have a significant effect on virus growth *in vitro* nor in virus virulence *in vivo*.

## Results

### *Generation of FMDV 3B mutants*

Using a full-length FMDV A24-WT cDNA clone (Rieder et al., 2005), nine plasmids containing 19mer inserts in the 3B region were generated by random transposon mediated insertion mutagenesis. Mutated plasmids were completely sequenced to confirm the exact insertion site. RNA derived from each of nine plasmids containing individual insertions in each of the 3B proteins were use for electroporation of BHK-21 cells giving origin to five viruses showing either insertions or deletions in 3B and additional changes elsewhere in the genome after amplification in BHK-21 cells (Table 1). Viruses derived from plasmids pA24-$VPg_1$-5873, pA24-$VPg_2$-5934 and pA24-$VPg_3$-6007 harbored individual inserts in one of their 3B proteins and were named A24-$VPg_1$-5873, A24-$VPg_2$-5934 and A24-$VPg_3$-6007 respectively (Fig. 1A). Two plasmids with inserts in $3B_1$ (pA24-$VPg_1$-5853 and pA24-$VPg_1$-5869) yielded viruses that lost the original insert along with one full 3B, including the 3′ end of $3B_1$ and the 5′ end of $3B_2$ resulting in mutant viruses A24-ΔVPg-5853 and A24-ΔVPg-5869, respectively (Fig. 1B). The full-length genome of the five mutant viruses was sequenced and showed very few differences throughout the genome besides their 3B insertions or deletions (Table 1).

### *Cell growth characteristics of FMDV 3B mutants*

The growth kinetics of the five mutant viruses was compared with the parental virus A24-WT in EBK cells (primary embryonic bovine kidney cells). Cell cultures were infected with each of the viruses at high and low multiplicity of infection (MOIs) (5, 0.1 and 0.01). At different time points post-infection samples were taken and titrated on BHK-21 cells. At all MOIs mutant viruses demonstrated growth kinetics similar to that of the parental virus (Fig. 2). All viruses obtained at 24 h time point from the growth curve generated with an MOI of 0.01 were full-length sequenced to confirm that the original insertions and deletions were still present in a mostly homogeneous viral population. No changes were detected in any of the viruses analyzed (results not shown).

Plaque morphology of the FMDV 3B mutant viruses was evaluated on five FMDV susceptible cell lines: BHK-21 (hamster kidney-derived cells), BHK- $\alpha_V\beta_6$ (BHK-21 cells constitutively expressing bovine integrins (Duque et al., 2004)), IBRS2 (swine-derived cell line), LFBK (bovine-derived cell line (Swaney, 1988)) and EBK. No significant differences in the titer (Fig. 3A) or plaque sizes were found, with the exception of A24-$VPg_3$-6007 that showed a 40–50% smaller plaque size in BHK-21, BHK-$\alpha_V\beta_6$ and EBK cells, but not in LFBK or IBRS2 cells (Fig. 3B).

### *Kinetics of viral RNA synthesis in FMDV with altered 3B proteins*

The effect of modifications in 3B proteins on viral RNA synthesis during infection of BHK-21 and EBK cell was analyzed. Cultures were infected with each of the mutant or parental A24-WT viruses at an MOI of 5 and 0.05. Total intracellular FMDV RNA concentrations were determined by quantitative rRT-PCR (Callahan et al., 2002) at different sampling points during the first 4 h post-adsorption. Mutant viruses showed similar pattern of RNA synthesis to A24-WT at both low and high MOI in both cell types. Only a small delay in RNA synthesis at 1–2 h post-adsorption in BHK-21 cells was observed in mutant A24-$Vpg_3$-6007 (Fig. 4).

### *Processing of 3A and 3B proteins in FMDV 3B mutants*

Since 3B is too small to be readily resolved by standard polyacrylamide gel electrophoresis, its presence is usually visualized in partially processed intermediates (Falk et al., 1992; Pacheco et al., 2003). Top (I) and center (II) portions of Fig. 5 show a Western blot developed using a rabbit polyclonal serum that recognizes the conserved N-terminal portion of 3A (O'Donnell et al., 2001). The bottom portion (III) of Fig. 5 shows reactivity with a pool of monoclonal antibodies against 3B to confirm the assignment of products made on the top and center portions of the figure. A typical A24-WT electrophoretic pattern shows five 3A-3B proteins and the mobility observed for these proteins was slightly slower than predicted, as previously reported (Garcia-Briones et al., 2006; O'Donnell et al., 2001; Pacheco et al., 2003; Strebel et al., 1986). The fastest migrating band (band 1) represents degraded-3A (Pacheco

**Table 1**
Nucleotide and amino acid sequence of mutant viruses obtained after transfection or from clinical samples.

| Plasmid | Virus obtained in cell culture | Nucleotide and amino acid changes related to FMDV A24-WT[c] | | | | | | | Partial sequence (3B region) of viruses obtained from infected animals[f] |
|---|---|---|---|---|---|---|---|---|---|
| | | 5'UTR[d] | | VP1 | 3D | | | | |
| | | 542[e] | 924 | 3730 | 6752 | 6952 | 7041 | 7294 | |
| pA24-WT | FMDV A24-WT | A | T | C (Ala) | C (Leu) | T (Val) | A (Glu) | T (Pro) | |
| pA24-$VPg_1$-5853 | A24-ΔVPg-5853[a] | A | T | T (Ala) | T (Leu) | C (Val) | A (Glu) | T (Pro) | No change in sequence |
| pA24-$VPg_1$-5869 | A24-ΔVPg-5869[b] | A | T | C (Ala) | T (Leu) | T (Val) | A (Glu) | C (Pro) | No change in sequence |
| pA24-$VPg_1$-5873 | A24-$VPg_1$-5873 | A | - | C (Ala) | T (Leu) | T (Val) | C (Ala) | T (Pro) | No change in sequence or deletion at nucleotides 5842–5878 and 5936–5967[g] |
| pA24-$VPg_2$-5934 | A24-$VPg_2$-5934 | C | T | C (Ala) | T (Leu) | T (Val) | A (Glu) | T (Pro) | No change in sequence |
| pA24-$VPg_3$-6007 | A24-$VPg_3$-6007 | A | T | C (Ala) | T (Leu) | T (Val) | A (Glu) | T (Pro) | No change in sequence |

[a] Virus with deletion at nt 5829–5897.
[b] Virus with deletion at nt 5856–5924.
[c] Full-length genome sequencing of 6 high titer stock viruses was performed.
[d] Virus region.
[e] Nucleotide position.
[f] Partial sequence including only the 3B region of viruses obtained from cows during the acute stage of the disease (days 4–6 post-inoculation) to confirm non-reversion to wild-type.
[g] Viruses from clinical samples collected from this cow were partially sequenced and they either retained the insert in 3B or contained deletions of the 3′ end of $3B_1$ and 5′ end of $3B_2$, resulting in a virus similar to the A24-ΔVPg viruses.

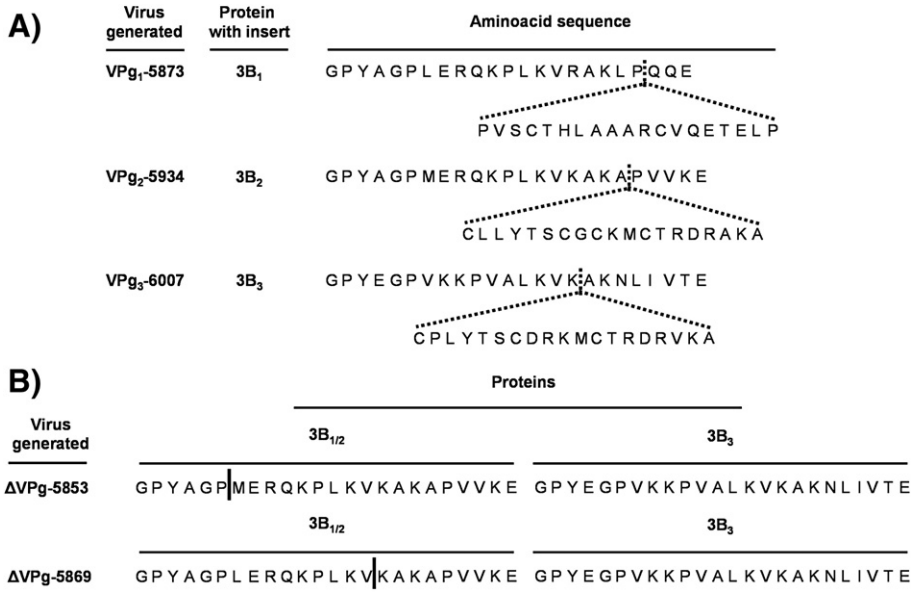


**Fig. 1.** Diagram of FMDV A24-3B mutants named based on the location of the mutation in pA24-WT. (A) Transposon-containing mutants with altered 3B protein shown for each virus. Remaining 3B amino acid sequences are the same as the parental virus (A24-WT). The amino acid at which the insertion occurred is marked with the dotted line. (B) Deletion-containing mutants. Solid line indicates where deletion took place and separates original regions of $3B_1$ (to the left) and $3B_2$ (to the right) in the resulting viruses.

et al., 2003) and the remaining four bands correspond to 3A (band 2), $3AB_1$ (band 3), $3AB_1B_2$ (band 4) and $3AB_1B_2B_3$ (band 5). In order to better visualize several weak bands a longer exposure of the same gel is shown in top portion of Fig. 5. Doublets observed (e.g. bands 3 and 4) could result from incomplete reduction of these proteins (Garcia-Briones et al. (2006)) or from uridylation of the 3B portion of the proteins or unspecific protease activity as previously described by Strebel et al. (1986). All 3B containing bands (3, 4 and 5) were also visualized with anti-3B antibodies (Fig. 5, panel III). The two mutants with one 3B deleted (A24-ΔVPg) showed the same bands 1 and 2 as A24-WT, and two bands very similar in size to bands 3 and 4 corresponding to $3AB_{1/2}$ (band 6) and $3AB_{1/2}B_3$ (band 7) respectively. As expected, a band corresponding to the form with three 3Bs (band 5) was absent in these viruses as confirmed in longer film exposures (top portion). In these two mutants only one 3B band was detected by anti-3B antibodies, suggesting that the $3AB_{1/2}B_3$ (band 7) is not recognized

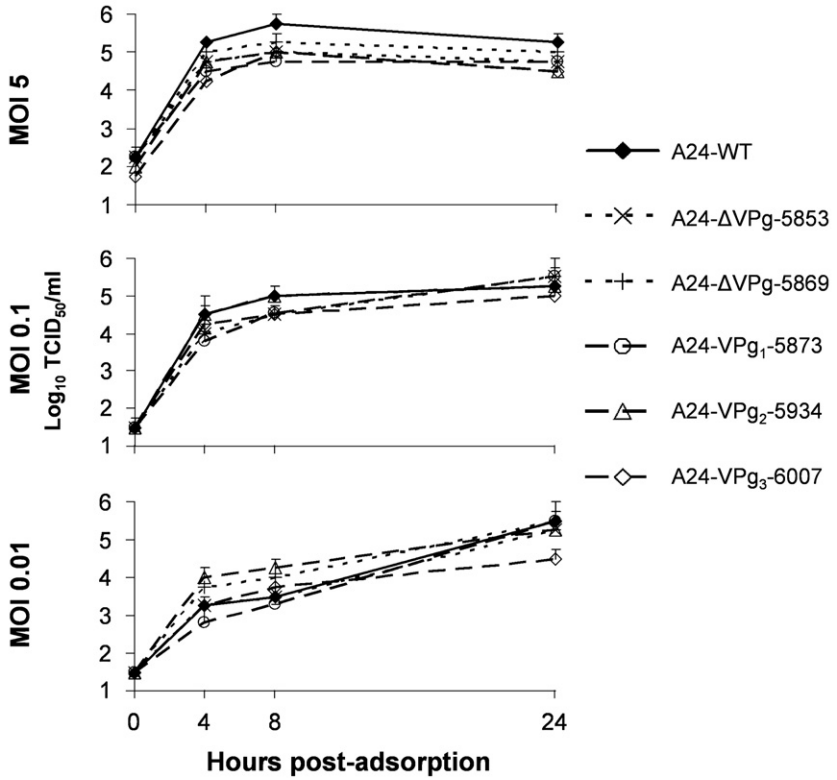


**Fig. 2.** Multi-step growth curve of either A24-mutants or A24-WT viruses. EBK cell cultures were infected at an MOI 5 (top panel), 0.1 (center panel) or 0.01 (bottom panel). Virus titers were performed on BHK-21 cells and expressed as $TCID_{50}$/ml on BHK-21 cells. Values are averages and standard deviations representative of two independent experiments.

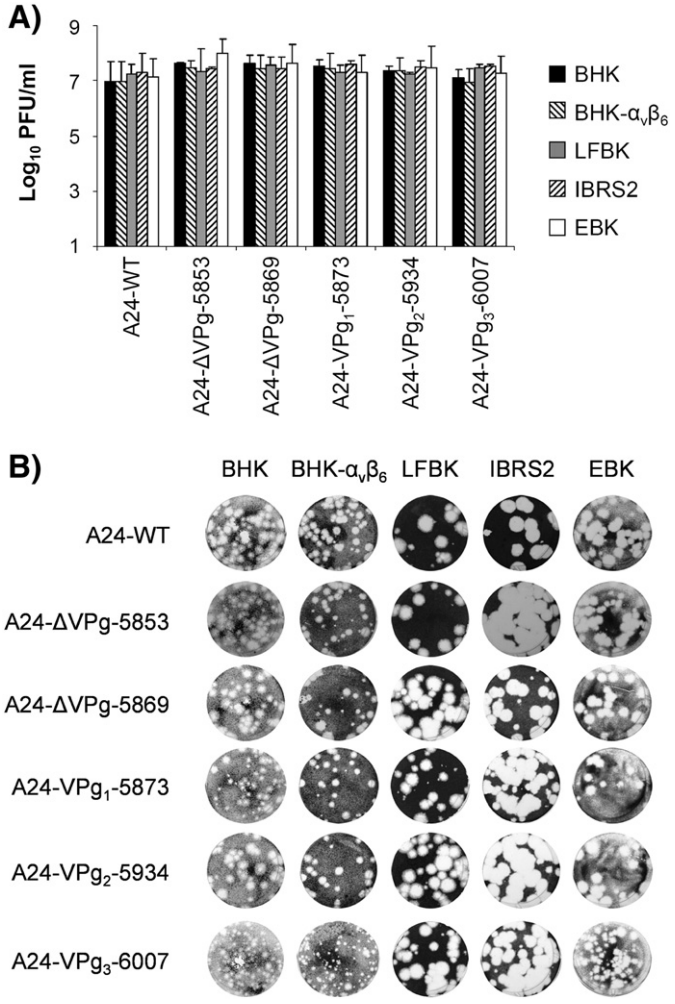


**Fig. 3.** (A) Ability of the FMDV A24 3B mutant viruses to form plaques in cell cultures derived from different animal species. PFU/ml values were determined starting with viruses at dilutions containing $10^7$ PFU/ml, as previously determined in BHK-21 cells. Values are averages and standard deviations representative of two independent experiments. (B) Plaque size assessment of either A24-mutants or A24-WT viruses on BHK-21, BHK- $\alpha_V\beta_6$, LFBK, IBRS2 and EBK cells. Cells were infected and after 1 h adsorption, 0.6% gum tragacanth overlay was added, followed 48 h later with crystal violet staining.

by these antibodies (Fig. 5, panel III). Virus A24-$VPg_1$-5873, containing an insert in $3B_1$, showed bands 1 and 2 without modifications and a single band (identified as band 8) co-migrating with band 3 ($3AB_1$) of A24-WT. We expected band 8 to be a product of larger size than band 3 due to the presence of the transposon insert, but a putative 3C cleavage site (www.expasy.org/tools/peptidecutter) present within the 3′ end of the insert itself may have resulted in a 3AB product similar in size to wild-type $3AB_1$. Additionally for this virus, differences were found with respect to A24-WT in the processing of the 3′ end of P3 after the transposon (bands 9 and 10). However, all 3B containing putative bands (8, 9 10) were detected by anti-3B antibodies (Fig. 5, panel III). Virus A24-$VPg_2$-5934 showed bands 1–3 as A24-WT and band 11 co-migrating with band 4. Again, we expected a larger $3AB_1B_2$ due to the presence of the transposon insert in $3B_2$, but putative 3C cleavage sites within the transposon might explain the smaller than expected $3AB_1B_2$ (band 11). Similar to what is described for A24-$VPg_1$-5873, the 3′ end of P3 showed a different pattern than A24-WT (bands 12 and 13) but all putative 3B containing bands reacted with anti-3B (Fig. 5, panel III). Virus A24-$VPg_3$-6007 showed bands 1–4 same as A24-WT, band 14 likely formed by cleavage within the transposon and $3AB_1B_2B_3$-19mer and an unknown processing pattern for the 3′ end of P3 (band 15), all reactive with anti-3B antibodies (Fig. 5, panel III). Mock inoculated cells confirmed the specificity of the antibodies used in the Western blot. When analyzed with a pool of monoclonal antibodies against 3C no bands were detected, indicating that cleavage at site $3B_3$/3C is not affected by the presence of the insert or deletions (results not shown). Taken together, these results indicate that with the presence of deletions or insertions in 3B, mutant FMD viruses yield different 3AB protein products in infected BHK-21 cells.

*Assessment of FMDV 3B mutant virulence in cattle*

Virulence of mutants and parental A24-WT was assessed utilizing a well established aerosolization inoculation method that resembles natural infection (Pacheco et al., 2008). Cows were inoculated with $10^7$ $TCID_{50}$ of mutant or parental A24-WT and viral infection and clinical signs were monitored daily (Pacheco et al., 2008). Results demonstrated that all five animals, each inoculated with one of the mutant viruses developed clinical FMD as did the A24-WT infected animal and all reached a maximum or close to the maximum clinical score by the end of the study (9 days post-infection (dpi). However, fever was sporadic and the appearance of clinical signs was delayed in animals inoculated with 3B mutants compared to the animal inoculated with A24-WT. Interestingly, while viremia lasted three days in the cow inoculated with FMDV A24-WT, as well as in cows inoculated with mutants A24-ΔVPg-5869 and A24-$VPg_1$-5873, cows inoculated with the three remaining mutant viruses (A24-ΔVPg-5853, A24-$VPg_2$-5934 and A24-$VPg_3$-6007), presented a viremia that lasted one or two days. Virus shedding (from saliva) was detected in all animals from 2 to 8 dpi, with the exception of the animal inoculated with A24-$VPg_2$-5934, where virus was detected in very low amounts only at 2 and 6 dpi. Serum neutralizing antibodies were detected in all animals starting at 5 or 6 dpi (Fig. 6). Table 1 shows partial genomic sequence of multiple viruses recovered from lesions in animals inoculated with 3B mutant viruses confirming that the disease was caused by mutant viruses and not by reversions to wild-type virus. The only exception was a virus from one of five samples obtained from the animal inoculated with A24-$VPg_1$-5873. This virus had not only lost the insert but also removed an entire 3B, a similar mutation previously seen with the two 3B deletion mutants described above (A24-ΔVPg-5853 and A24-ΔVPg-5869).

## Discussion

Among the picornaviruses, FMDV has a unique genomic feature: the presence of redundant copies of the 3B protein. This redundancy was analyzed in the past. Falk et al. reported that although not all three copies of 3B are needed to maintain infectivity, 3B copy number influences RNA synthesis and production of infectious FMDV particles in cell culture. Their 3B deleted mutants had reduced viral RNA synthesis levels after infection and the levels of viral RNA synthesis and infective particle formation were found to correlate with the number of functional 3Bs left in the mutant viruses. These authors suggested a direct correlation of 3B gene dosage and viral RNA synthesis, with a secondary effect on infective particle formation (Falk et al., 1992). In poliovirus, it was shown that the 3B uridylylation reaction may employ a 3B-containing precursor (3AB or 3BC (D)) rather than the processed 3B (Liu et al., 2007; Oh et al., 2009; Pathak et al., 2008). Based on these reports, it could be hypothesized that the presence of three 3Bs could benefit FMDV replication due to the additive presence of multiple 3B or 3B-precursors proteins.

To address the importance of 3B in viral virulence, particularly in cattle, one of the main natural FMDV hosts, we created viruses with insertions in each of the 3B proteins as well as viruses with only two 3Bs in the genetic backbone of FMDV A24-WT, a virus that is fully virulent in cattle. It is important to note that each 3B protein was disrupted by the insertion of an amino acid sequence (19mer) similar in size to a single 3B protein itself. Therefore it is expected that this insertion severely altered the structure and affected the function of the individual 3B.

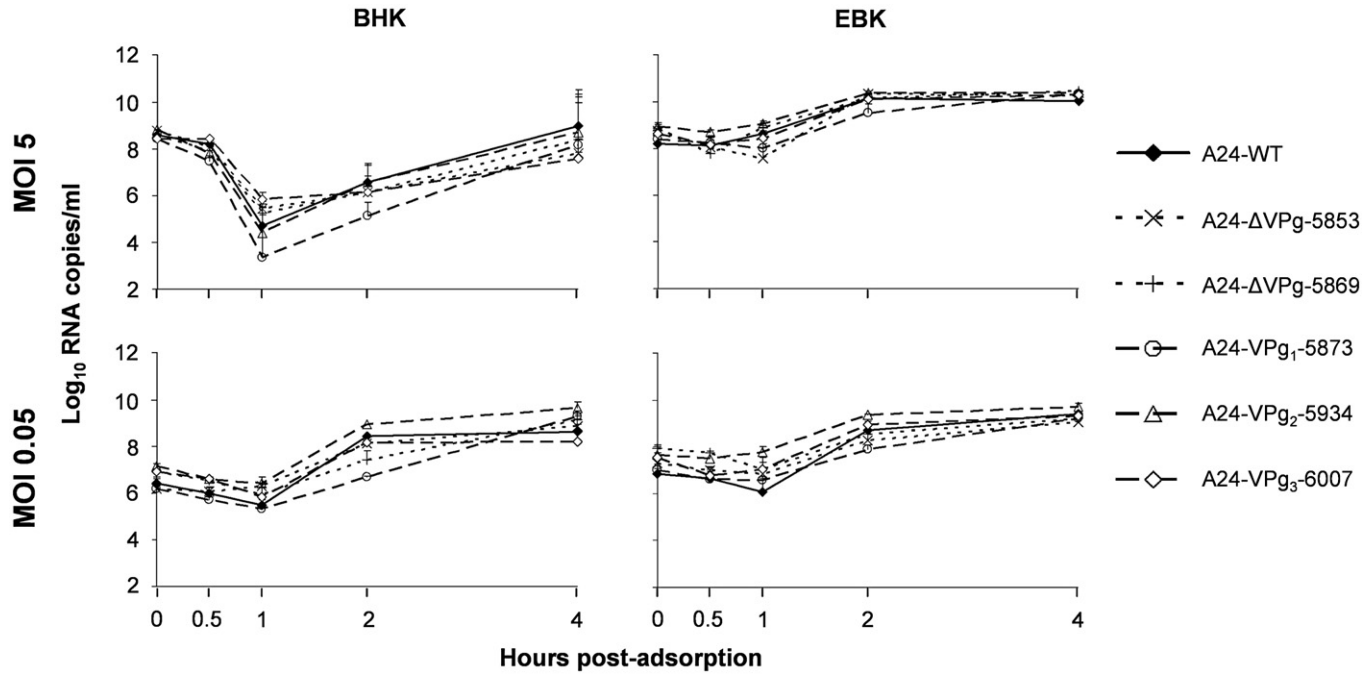


**Fig. 4.** Synthesis of viral intracellular RNA in cell cultures infected with either FMDV A24-3B mutants or parental virus. Values are expressed as amounts of RNA copy numbers recovered from BHK-21 and EBK cell cultures infected at an MOI of 5 or 0.05, and quantified by rRT-PCR. Data represents values and error bars from data obtained in an independent experiment. Experiments were reproduced at least three times.

In previous work it was shown that modifications in viral proteins 3A and 3B could decrease RNA synthesis and virus yield in different cell lines, particularly in bovine-derived cells (O'Donnell et al., 2001; Pacheco et al., 2003). It has also been described that expression of 3AB proteins resulted in an increase of FMDV replication *in vitro*, suggesting that the redundancy of 3B may be related with simultaneous presence of 3AB and 3B that would improve FMDV replication and translation (Rosas et al., 2008). Nevertheless, here we could not find significant differences in the level of RNA synthesis among all mutants when compared with the parental virus. Similar results were obtained when the growth abilities of the mutant viruses were analyzed in single or multistep growth curves. Thus, in our hands, severe domain disruptions in individual 3B proteins did not significantly affect the ability of FMDV to synthesize viral RNA nor to replicate and grow in susceptible cell lines.

Each of the three distinct FMDV 3B peptides is an efficient substrate in the FMDV VPgpU(pU) uridylation reaction, but $3B_3$ has been shown to have the highest activity (Nayak et al., 2005). Although it has been reported that modifications of the $3B_3$ coding sequence result in production of a noninfectious RNA transcript (Falk et al., 1992), we were able to derive a viral mutant containing a severe disruption of $3B_3$ (A24-Vpg$_3$-6007), albeit this virus showed a slight delay in viral growth.

It has been shown that Tyr-3 of 3B is critical in the process of poliovirus replication (Kuhn et al., 1988a,b; Liu et al., 2007; Paul et al., 2003; Reuer et al., 1990). Consistent with these previous results, our plasmid constructs containing inserts near the Tyr-3 residue of $3B_2$ or $3B_3$ (pA24-VPg$_2$-5887, pA24-VPg$_2$-5889, pA24-VPg$_2$-5949 and pA24-VPg$_3$-5974), yielded viruses that had reverted to wild-type genotype or did not yield viable progeny, despite the fact that the other two 3Bs were not affected (not shown).

To determine whether insertions or deletions in the individual 3Bs altered the processing of the P3 region, we analyzed the proteolytic cleavage products of the five mutant viruses *in vitro*. All 3B mutants maintained processing of P3 region (3A, 3B and 3C (not shown)) although an altered pattern of 3B protein processing was observed in some mutant viruses. Our proposed interpretation of the band patterns observed in Fig. 5 was based on the size and reactivity of these bands with specific anti-3A and anti-3B antibodies and previous studies documenting this processing (Pacheco et al., 2003).

In previous work it was reported that FMDV containing only one of the 3B proteins was partially attenuated when inoculated in swine (Pacheco et al., 2003). It has been suggested that there is an association between 3B copy number and virulence *in vivo* and this may explain why three copies of 3B are present in all FMDV strains characterized to date (Carrillo et al., 2007; MacKenzie et al., 1975). In contrast, when viruses were analyzed *in vivo* in this study, virulence was not drastically decreased in the 3B mutants compared to the parental wild-type FMDV except for some minor delays in the appearance of viremia and clinical signs and extent of shedding. Therefore, in our hands FMDV appears to have considerable flexibility in accepting insertions and deletions of the 3B region. Our results indicate that as long as two functional 3Bs (viruses A24-ΔVPg-5853 and A24-ΔVPg-5869) were maintained, the virus was able to replicate *in vitro* and cause disease in cattle. Although our data might suggest that two copies of 3B are sufficient for FMDV virulence, the minor delays in establishing infection and decreased shedding may be detrimental during the natural transmission cycle preventing these mutations to be maintained. Interestingly the mutants with 3B deletions (A24-ΔVPg-5853 and A24-ΔVPg-5869) combined parts of $3B_1$ and $3B_2$ while always conserving $3B_3$ intact. This was also true in the deletion mutant virus recovered from a cow inoculated with virus A24-VPg$_1$-5873. This could be due to the similarity of the first two 3Bs or to the critical need for $3B_3$ for adequate FMDV replication, as has been previously suggested (Nayak et al., 2005).

In summary we present data showing that severe domain disruptions of individual 3B proteins do not drastically affect FMDV RNA synthesis, virus replication or virulence in cattle. It is remarkable that the introduction of a 19 amino acid insert in a 23–24 amino acid protein does not appear to interfere with the basic viral activity during *in vitro* replication or even causing disease in cattle. The independence of FMDV virulence from the presence of three intact 3B proteins is difficult to reconcile with the conserved presence of these three proteins across hundreds of FMDV strains in all seven serotypes sequenced to date, suggesting that the redundancy of these genomic elements is required for viral processes not measured in this study.

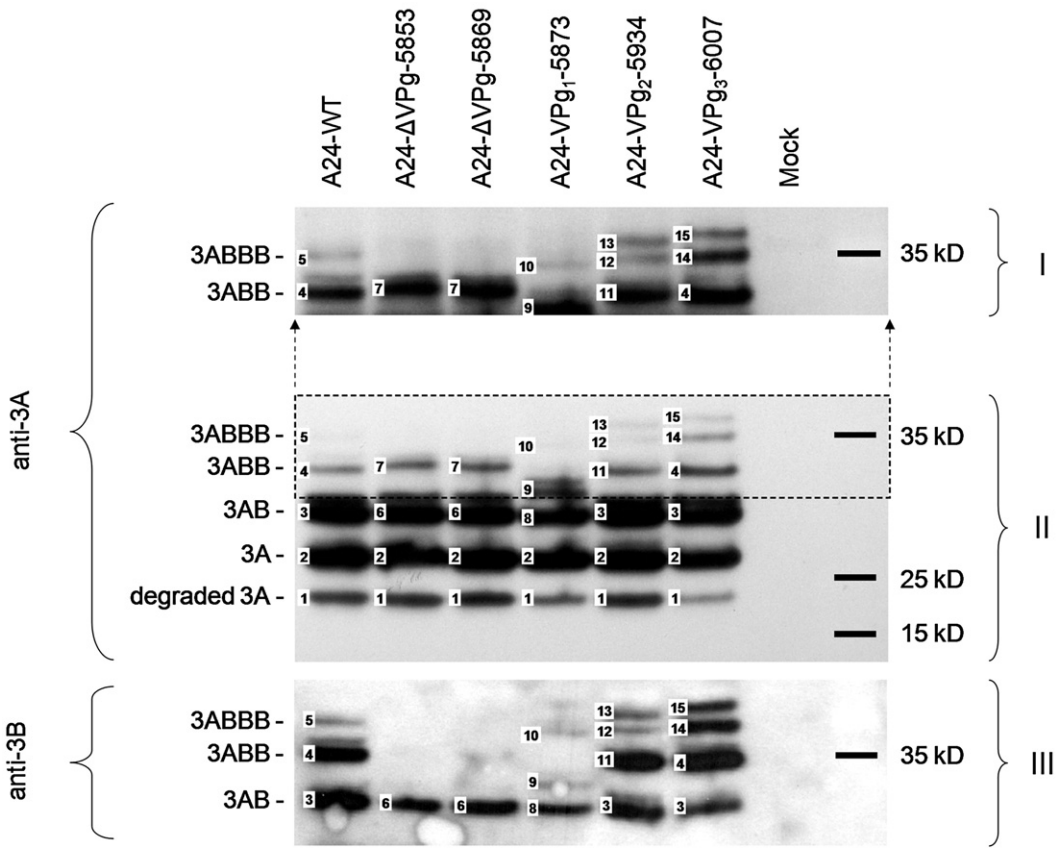


**Fig. 5.** Western blot to detect 3A and 3B proteins. Lysates of BHK-21 cells infected with either A24-3B mutants or parental virus were separated on SDS-PAGE. Western blotting was performed using a specific rabbit polyclonal serum against the N-terminal portion of 3A of FMDV-A12 (panels I and II) or monoclonal antibodies against FMDV 3B (panel III).

## Materials and methods

### *Construction of transposon-containing FMDV mutants*

To identify virulence determinants within the FMDV genome, we constructed a library of infectious FMDV full-length genome plasmids of pA24Cru (Rieder et al., 2005) containing single random insertions (1 per genome), using the EZ-Tn5™ Insertion Kit (Piccone et al., 2009; Piccone et al., 2010). Plasmids containing a 57 nt insertion in the 3B encoding region were selected for further study. Each of these plasmids contained an insertion at residues 5853, 5869, 5873, 5887, 5889, 5934, 5949, 5974 or 6007 and were named according to the mutation location in 3B, as pA24-VPg$_1$-5853, pA24-VPg$_1$-5869, pA24-VPg$_1$-5873, pA24-VPg$_2$-5887, pA24-VPg$_2$-5889, pA24-VPg$_2$-5934, pA24-VPg$_2$-5949, pA24-VPg$_3$-5974 and pA24-VPg3-6007, respectively. All nucleotide designations are based on the nucleotide sequence of A24 Cruzerio. T7 RNA transcripts of *SwaI*-linearized plasmids, including pA24Cru, were produced using a MegaScript T7 Kit (Ambion, Austin, Texas) followed by subsequent transfection into BHK-21 cells by electroporation as described previously (Piccone et al., 1995; Rieder et al., 1993). Mutant viruses obtained from these transfections were named according to the site of insertion (see below).

### *Cells and viruses*

Viruses containing single in-frame insertions or deletions in the 3B region were derived from the mutant plasmids and the A24-WT described above. In order to maintain adequate bovine receptor usage, virus stocks were grown in a derivative of baby hamster kidney (BHK-21) (baby hamster kidney cell line, ATCC, catalogue number CCL-10) cells expressing the bovine $\alpha_V\beta_6$ integrin (BHK-$\alpha_V\beta_6$) (Duque et al., 2004) and titrated on BHK-21 cells by calculating the 50% tissue culture infectious dose per ml ($TCID_{50}$/ml). Two of the plasmids yielded viruses containing one insert in 3B namely A24-VPg$_2$-5934 and A24-VPg$_3$-6007. Two other plasmids gave origin to viruses that lost the insert and a full 3B and these viruses were named A24-ΔVPg-5853, A24-ΔVPg-5869, respectively. After several attempts, the remaining five plasmids (pA24-VPg$_1$-5873, pA24-VPg$_2$-5887, pA24-VPg$_2$-5889, pA24-VPg$_2$-5949 and pA24-VPg$_3$-5974) gave no progeny or yielded viruses that had reverted to wild-type genotype when grown in BHK-$\alpha_V\beta_6$ cells with subsequent culturing in BHK-21 cells. The only virus with an insert obtained in a second round of transfections was A24-VPg$_1$-5873. Virus stocks were prepared with A24-WT, A24-ΔVPg-5853, A24-ΔVPg-5869, A24-VPg$_1$-5873, A24-VPg$_2$-5934 and A24-VPg$_3$-6007 and the complete viral sequence was determined using FMDV-specific primers designed to cover the entire genome. High titer stocks were determined in BHK-21 cells. *In vitro* phenotype was characterized by plaque assays in BHK-21 cells, BHK-$\alpha_V\beta_6$ cells, a bovine kidney cell line (LFBK) (Swaney, 1988), a porcine kidney cell line (IBRS2) (de Castro, 1964) and primary embryonic bovine kidney cells (EBK, kindly provided by Dr S. Wessman, USDA, APHIS, AMES, Iowa).

### *Comparative ability to grow and to form plaques*

In order to characterize *in vitro* growth, plaque assays were performed in the 5 different cell types described above (BHK-21, BHK-$\alpha_V\beta_6$, IBRS2, LFBK and EBK cells) with the 6 selected viruses described above (FMDV A24-WT, A24-ΔVPg-5853, A24-ΔVPg-5869, A24-VPg$_1$-5873, A24-VPg$_2$-5934 and A24-VPg$_3$-6007) to compare plaque size and ability to replicate in the different cell types. The ability of these viruses to replicate in different cell types was determined by testing serial 10-fold dilutions of virus (starting with $10^7$ PFU/ml [as measured previously on BHK-21 cells]) to form plaques on these different cells.

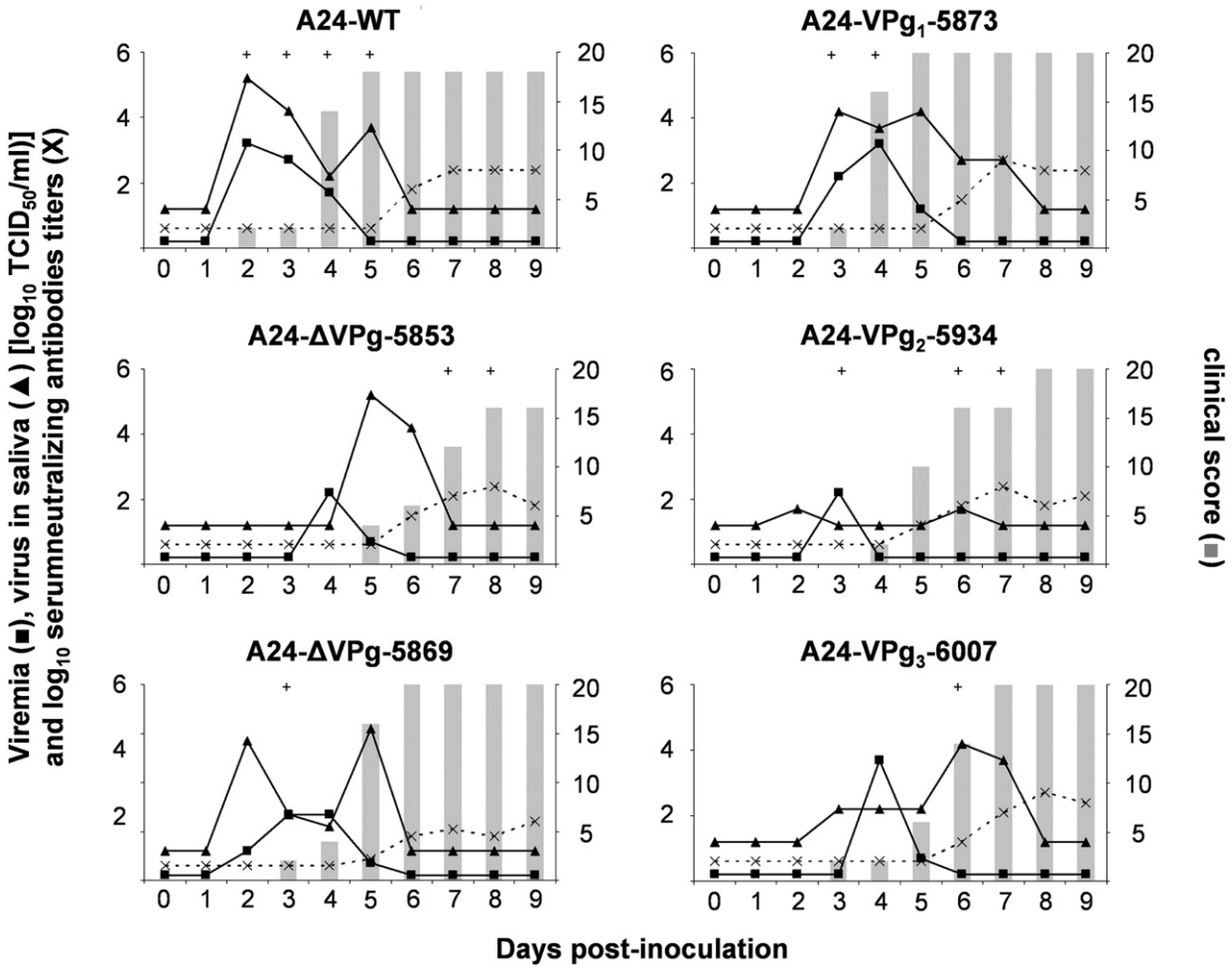


**Fig. 6.** Comparison of disease induced in cattle by either A24-3B mutants or A24-WT viruses. Animals were inoculated with aerosol containing $10^7$ $TCID_{50}$. Viremia (■), virus in saliva (▲) and serum-neutralizing antibody titers (X) are expressed in the left axes; the clinical score (bars) are expressed in the right axes. + denotes fever (>40 °C).

Briefly, preformed 95% confluent monolayers were inoculated with dilutions of each virus. Tragacanth gum overlay (0.6%) was added after 1 h adsorption and after 48 h the plates were fixed and stained with crystal violet. Analyses were performed side-by-side with BHK-21 cells, and the resulting data was used to calculate the PFU/ml. All samples were run simultaneously to avoid inter-assay variability.

*Virus growth curve in primary tissue culture*

Growth curves were performed in EBK cells. Preformed monolayers were prepared in 24-well plates and infected with the six viruses described above (A24-WT, A24-ΔVPg-5853, A24-ΔVPg-5869, A24-$VPg_1$-5873, A24-$VPg_2$-5934 and A24-$VPg_3$-6007) at MOIs of 5, 0.1 and 0.01 (based on $TCID_{50}$ previously determined in BHK-21 cells). After 1 h of adsorption at 37 °C the inoculum was removed and the cells were rinsed two times with ice-cold 145 mM NaCl, 25 mM MES (pH 5.5) to remove residual virus particles. The monolayers were then rinsed with media containing 1% fetal calf serum and 25 mM Hepes (pH 7.4) and incubated for 0, 4, 8 and 24 h at 37 °C. At appropriate times post-infection, the cells were frozen at −70 °C and the thawed lysates were used to determine titers by $TCID_{50}$/ml on BHK-21 cells.

*Determination of intracellular viral RNA concentration*

BHK-21 and EBK cells were seeded in 24-well plates, infected at MOIs of 0.5 and 5 with all 6 viruses (A24-WT, A24-ΔVPg-5853, A24-ΔVPg-5869, A24-$VPg_1$-5873, A24-$VPg_2$-5934 and A24-$VPg_3$-6007), adsorbed and acid washed as described above. After 0, 0.5, 1, 2 and 4 h incubations at 37 °C, cell supernatants were discarded and total cellular RNA was extracted from monolayers with Lysis/Binding Solution (Ambion, Austin, TX), then frozen until further use. Quantitation of FMDV RNA was performed by rRT-PCR as previously described (Pacheco et al., 2008). Three separate experiments were performed and CT values converted to the number of RNA copies/ml based on FMDV RNA specific calibration curves developed with *in vitro*- synthesized RNA.

*Western blot analyses of 3A and 3B polypeptides in infected cells*

BHK-21 cells were infected with every mutant and parental A24 viruses at an MOI of 5 and when 60–70% of the cells displayed CPE (approximately 6 hpi) the supernatant was discarded and the cell monolayers were lysed with TNET buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% [wt/vol] Triton X-100) for 20 min on ice; after clarification, the cell lysates were stored at −70 °C. Samples were resolved by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels (NuPAGE® Novex® Bis-Tris Mini Gels; Invitrogen) and electroblotted onto polyvinylidene difluoride membranes (Millipore) by standard methods. FMDV-specific proteins were detected with a chemiluminescent Western Blot immunodetection kit (WesternBreeze®, Invitrogen). For detection of the different forms of 3AB, a polyclonal rabbit serum generated by using an Escherichia coli-expressed N-terminal fragment of 3A of FMDV-A12 (O'Donnell et al., 2001) diluted 1/500 was used as the primary antibody. For detection of 3B a pool of monoclonal antibodies was used as the primary antibody diluted 1:500 (monoclonals 1G7 and 1A10,

kindly provided by Dr. E. Brocchi). For detection of 3C a pool of monoclonal antibodies was used as the primary antibody diluted 1:500 (monoclonals 2B7 and 1H6, kindly provided by Dr. E. Brocchi).

### *Viral virulence in cattle, serology and virus isolation*

Cattle experiments were performed under Biosafety level 3 conditions in the animal facilities at PIADC following a protocol approved by the Institutional Animal Use and Care Committee. Steers (300–400 kg) were infected using an aerosolization method that resembles natural infection (Pacheco et al., 2008). Briefly, animals were sedated and inoculated with $10^7$ $TCID_{50}$ in 2 ml of MEM delivered by aerosol with a jet nebulizer (Whisper Jet, Marquest Medical Products, CO) attached to an aerosol delivery system (Equine Aeromask, Medium Size, Trudell Medical, London, ON, Canada). One steer per virus (FMDV A24-WT, A24-ΔVPg-5853, A24-ΔVPg-5869, A24-$VPg_1$-5873, A24-$VPg_2$-5934 and A24-$VPg_3$-6007) was inoculated and held in individual animal rooms. Steers were clinically examined, including rectal temperature recordings, for 9 days after inoculation, and then humanely euthanized. Serum and oral swabs were collected and cows were sedated daily for clinical evaluation. After collection, samples were aliquoted and frozen at −70 °C. Neutralizing antibody titers in serum were determined as described elsewhere (Golde et al., 2005). Briefly, serial dilutions of serum were incubated with a virus dose of 100 $TCID_{50}$ of FMDV A24 for 1 h at 37 °C and then BHK-21 cells were added on top and incubated at 37 °C for 48–72 h. End-point titers were calculated as the reciprocal of the last serum dilution to neutralize 100 $TCID_{50}$ of virus in 50% of the wells. One serum and one swab aliquot were used to perform viral titration on BHK-21 cells by calculating the 50% tissue culture infectious dose per ml ($TCID_{50}$/ml) as described elsewhere (Pacheco et al., 2003). Clinical scoring, with a maximum of 20, was performed based on severity of lesions on the four feet, the snout, and the mouth as described elsewhere (Pacheco et al., 2008).

## Acknowledgments

We thank Elizabeth Bishop, Ethan Hartwig and George Smoliga for technical assistance. We would particularly wish to thank Melanie Prarat for editing the manuscript. We also thank the PIADC animal care staff for excellent technical assistance. This research was funded by ARS-CRIS Project 1940-32000-052-00D.

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